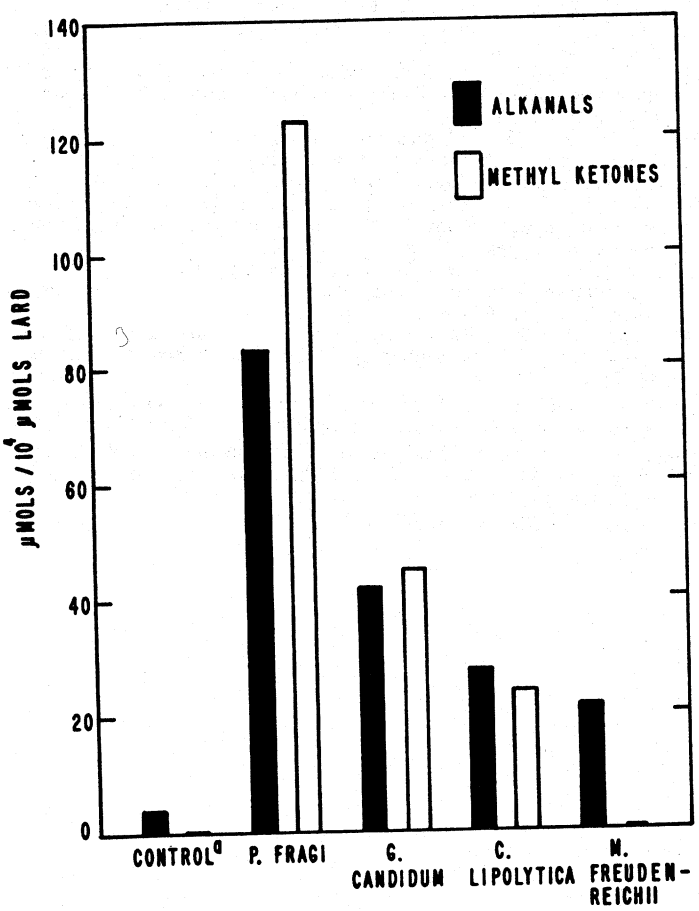

Fig. 3. Effect of microorganisms on alkanals and methyl ketones in fresh lard. [a]All microorganisms listed in Fig. 1, other than the four shown here had no effect.

activity [Lipoxygenase, EC 1.13.1.13 (Intern. Union Biochem., 1965)]. Tappel (1963) stated that there is no evidence for the presence of lipoxidase (that is, an enzyme similar to plant lipoxidase) in microorganisms. However, several reports suggest that a lipoxidase may be found in *Aspergillus, Penicillium, Rhizopus, Pseudomonas,* and *Achromobacter* (Mukherjee, 1951; Fukuba, 1952; Shimahara, 1966).

The three microorganisms which produced large increases in methyl ketones (Fig. 3) caused no accumulation of peroxides. Hawke (1966) reviewed the production of methyl ketones by microorganisms and cited several workers who had shown that lipolytic fungi growing in fat-containing media liberate fatty acids which are converted into β-keto acids and subsequently decarboxylated into methyl ketones. This may explain the production of methyl ketones by the three organisms in the present study since they were also active lipase producers. The ability of *A. flavus* to produce methyl ketones in rancid but not in fresh lard suggests that it is forming ketones by a route other than the β-keto acid mechanism. The mold, although lipolytic, does not produce methyl ketones unless peroxides are present.

Castell *et al.* (1941) and Jensen (1954) suggested that micro-organisms which are both active lipase-producers and oxidase-producers are responsible for rancidity in fat.

Table 1. Relationships of oxidase reaction, lipase production, and oxidative activity by microorganisms on lard.

	Lipase production				Oxidase reaction	
	µeq FFA/hr					
Effect on fresh lard	>50	10–50	1–10	0	+	−
Increase peroxides, 2-enals, 2,4-dienals, and alkanals	0[1]	0	1	0	0	1
Increase peroxide only	0	0	3	0	1	2
Increase alkanals and methyl ketones	2	1	0	0	2	1
Destroy peroxides, 2-enals, 2,4-dienals; alkanals unchanged	2	2	0	1	0	5
Destroy 2,4-dienals only	0	0	4	0	2	2
None	3	0	6	4	0	13

[1] Number of cultures.

The data in Table 1 do not support this conclusion. *M. freudenreichii,* the most active oxidizer of fat in this investigation, produced neither lipase nor oxidase. No correlation existed between ability to produce a lipase and production of a positive oxidase reaction, nor between either one or both of these enzymes and degree of fat oxidation. Likewise, there was no inverse relationship relating these two tests to the destruction or formation of peroxides and monocarbonyls. It is apparent that the "oxidase reaction" is not a measure of the glyceride fat oxidative ability of a culture. Use of this test should be limited to specific instances in which it is either of taxonomic value, as in the differentiation of *Neisseria* spp. or, as in gram-negative bacteria where a positive oxidase test may be indicative of the presence of cytochrome C (Baumann *et al.,* 1968).

When the activities of the microorganisms on fresh lard were compared with those observed on rancid lard, a striking difference was observed. On rancid lard, *A. flavus, Streptococcus lactis, Pediococcus cerevisiae, Lactobacillus dextranicum,* and *M. freudenreichii* produced definite increases in the concentrations of three aldehyde monocarbonyl fractions (Smith *et al.,* 1968), whereas only *M. freudenreichii* caused an increase in these fractions in fresh lard. Since there are essentially no peroxides in fresh lard, an organism that increases the concentration of any of the monocarbonyls would have to produce the peroxides from which they are formed. Since *M. freudenreichii* was the only one of these five to cause an accumulation of peroxides, this may explain why it was also the only one to show the monocarbonyl increase in fresh lard.

Obviously, the complexities of the pathways involved in the development of oxidative rancidity of fat are not reduced by the introduction of microorganisms into the system. Nevertheless, it is apparent that microorganisms can play an active role in both the development and the control of oxidative rancidity. As more evidence is obtained on the fat-degradation activity of specific microorganisms and on the specific conditions influencing their metabolism, useful control measures should evolve.

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Action of Microorganisms on the Peroxides and Carbonyls of Fresh Lard

SUMMARY—The effect of 28 microorganisms on the accumulation of hydroperoxides and monocarbonyls in fresh fat has been determined. *Pseudomonas ovalis, Micrococcus freudenreichii* and two strains of *Streptomyces* increased the concentration of peroxides; *M. freudenreichii* also produced an increase in 2-enals, 2,4-dienals, and alkanals. *Pseudomonas fragi, Geotrichum candidum,* and *Candida lipolytica* produced an increase in alkanals and methyl ketones. All other cultures either removed the small amount of peroxides and monocarbonyls in fresh fat and prevented any build-up or had no effect. The ability of microorganisms to oxidatively attack fats was not related to their oxidase reactions or their ability to produce lipase. Differences in oxidative activity on fresh and rancid lard are discussed.

INTRODUCTION

Lea (1938) and Jensen (1954), in reviewing the literature on development of rancidity in fats and fat-containing products, concluded that microorganisms could increase the rate of development of oxidative rancidity in fats. Recent studies from our laboratory (Smith *et al.,* 1968), however, have shown that the peroxides and carbonyls in fats made rancid by ultraviolet oxidation are often decreased by microbial action. The present investigation was undertaken to determine whether oxidative rancidity development in fresh lard would be enhanced or inhibited by microorganisms and whether differences existed among microorganisms.

METHODS AND MATERIALS

Preparation of fresh lard emulsion

Tween 60 was added to freshly rendered lard (in a ratio of 1/100), and emulsions containing 20% lard were prepared by treatment of 100-ml portions of the water-lard-Tween mixture with a Branson sonifier (Model S-125 set at maximum amplitude) for approximately 3 min. The resulting emulsion was autoclaved at 121°C for 15 min, cooled, and immediately added to the culture flasks.

Microorganisms

Twenty-eight strains of microorganisms, including bacteria, yeast and molds, were studied in this investigation. They were cultivated in static or aerated cultures as previously described (Smith *et al.,* 1968).

Action of microorganisms on fresh fat

When lipase production had reached its peak (or when cell growth was near the maximum if no lipase was produced), sterile fresh lard emulsion was added to the cultures so that the final concentration of lard was 3%. The culture-lard emulsion mixtures were incubated as stationary cultures at the optimum growth temperatures of the respective microorganisms for an additional 5 days. Uninoculated medium-lard mixtures were utilized as controls and were incubated at the same temperatures as the experimental flasks.

Assay of lipase

Lipase assays were determined as previously described (Alford *et al.,* 1963) with incubation at 35°C for one hr. All cultures were assayed at pH 7.0 except *Candida lipolytica, Aspergillus niger, Rhizopus oligosporus,* and *Thamnidium elegans,* which were assayed at pH 6.0.

Peroxide determination

The peroxide values were determined by the cold method of Lea (1952) using fat samples of 2 to 3 g.

Monocarbonyl determination

At the end of the incubation period, the culture-lard emulsion mixtures were centrifuged and the fat was extracted from the supernatant with petroleum ether (40 to 60°C b.p. range). The monocarbonyl fraction was isolated by column chromatography as described by Schwartz *et al.* (1962, 1963), and monocarbonyls were further separated into classes by the chromatographic techniques of Gaddis *et al.* (1959). The classes were identified by their R_f values on paper and by their absorption maxima utilizing a Cary recording spectrophotometer. In order to calculate concentrations on a molar basis, an average molecular weight of 1000 was assumed for the lard.

Oxidase test

The oxidase test was performed by adding a few drops of 1% N,N-dimethyl-*p*-phenylenediamine·HCl to microbial colonies present on Difco veal infusion agar plates. Oxidase positive colonies turned dark red (Kolmer *et al.,* 1951).

RESULTS

FRESH LARD CONTAINS peroxides and the same classes of monocarbonyls, i.e., 2,4-dienals, 2-enals, and alkanals, as those found in rancid lard. The concentrations are much lower, however, and the specific compounds within each group are not necessarily the same. Several determinations of the oxidative activity on lard by each culture were made and the percentage variation among runs with the same microorganism was always within 5 to 15%. The data presented in the figures are from representative experiments.

The effect of microorganisms on the peroxide accumu-

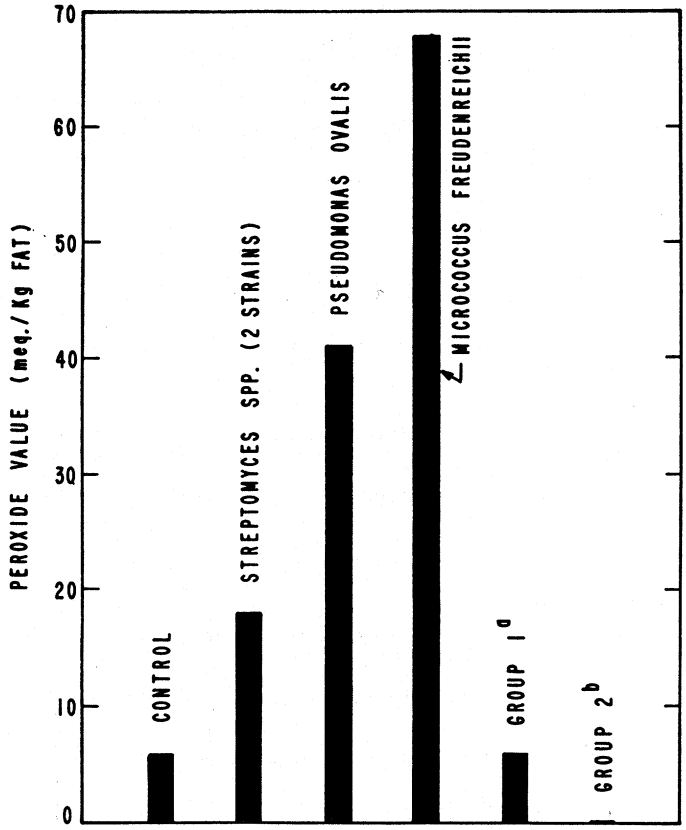

Fig. 1. Effect of microorganisms on peroxides in fresh lard. [a] *Group 1:* Escherichia coli, Streptococcus lactis, Streptococcus cremoris, Leuconostoc mesenteroides, Leuconostoc dextranicum, Leuconostoc citrovorum, Lactobacillus casei, Pediococcus cerevisiae, Sarcina lutea, Hansenula anomala, Thamnidium elegans, Penicillium roquefortii, Pseudomonas *sp.*, Aspergillus niger. [b] *Group 2:* Pseudomonas fragi, Staphylococcus aureus *(2 strains)*, Geotrichum candidum, Serratia marcescens, Rhizopus oligosporus, Bacillus cereus *(2 strains)*, Candida lipolytica, Aspergillus flavus.

lation in fresh lard is shown in Fig. 1. Four of the 28 microorganisms studied produced a definite increase in peroxides, whereas about half removed the small amount of peroxide found in the fresh lard. *Micrococcus freudenreichii* produced the highest concentration of peroxides.

Monocarbonyls, particularly 2,4-dienals, 2-enals, and alkanals, are commonly associated with the development of oxidative rancidity in fats. Among the 28 microorganisms, only *M. freudenreichii* caused an increase in the two unsaturated monocarbonyl classes. Fig. 2 shows that the other 27 cultures either removed or had no effect on the small amounts of one or both of these classes present in fresh lard.

Four microorganisms, *Pseudomonas fragi, Geotrichum candidum, Candida lipolytica* and *M. freudenreichii,* increased the concentration of alkanals (Fig. 3). The first three of these also produced considerable quantities of methyl ketones which are not found in detectable quantities in fresh or autoxidized lard.

Since lipase and oxidase production by microorganisms have been associated in the past with their ability to oxidize fats, the interrelationships of these activities for the 28 microorganisms were compared in this study as is shown in Table 1. Neither oxidase production as measured by the usual "oxidase reaction" nor ability to produce an active lipase was closely correlated with the observed action on the fat.

DISCUSSION

THE ACCELERATION of peroxide formation and the increase in concentration of three classes of monocarbonyls associated with fat oxidation that was produced by *M. freudenreichii* were unique among the 28 microorganisms examined. These data suggest that certain microorganisms, although relatively rare, can carry out the oxidative reactions usually associated with the oxidative rancidity development, viz., production of peroxides and subsequent decomposition into carbonyls. The other two organisms which produced an increase in peroxides did not show the subsequent accumulation of monocarbonyls. This indicates either (a) the mechanism [enzymatic or catalysis by heme-like compounds (Maier *et al.*, 1959)] for conversion of peroxides to monocarbonyls is not present in these organisms, (b) the monocarbonyls are subsequently destroyed as rapidly as they are formed, or (c) the peroxides are converted to dicarbonyls or other compounds which were not determined.

The ability of these three organisms to accelerate peroxide formation in a fat may well represent lipoxidase-like

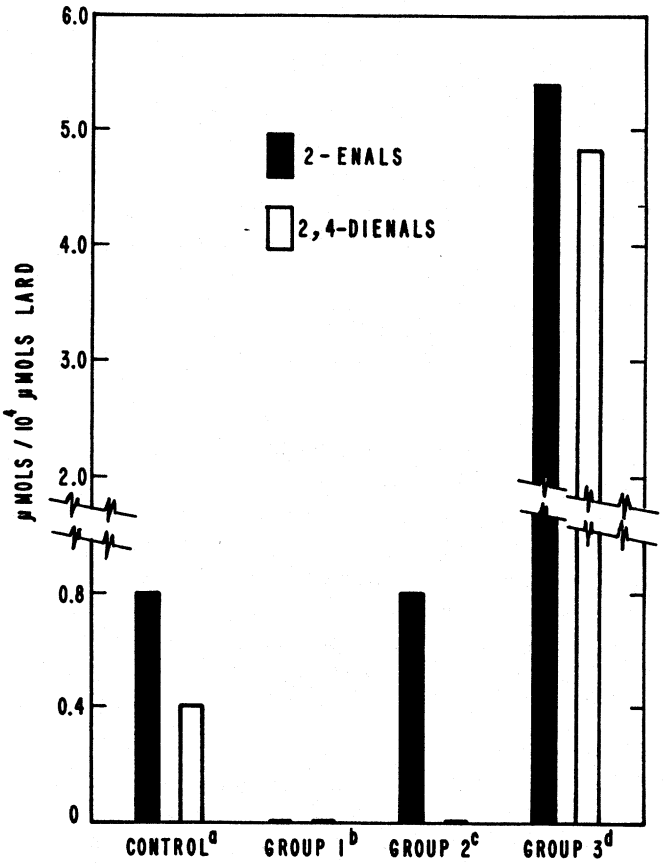

Fig. 2. Effect of microorganisms on 2,4-dienals and 2-enals in fresh lard. [a] *All microorganisms listed in Fig. 1 and not included in Groups 1, 2 or 3 had no effect on monocarbonyls.* [b] *Group 1:* P. fragi, G. candidum, C. lipolytica, S. aureus *(2 strains)*, B. cereus *(2 strains)*, A. niger. [c] *Group 2:* E. coli, S. marcescens, P. ovalis, Pseudomonas *sp.* [d] *Group 3:* Micrococcus freudenreichii.